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14. ABSTRACT

Aging is the single largest risk factor for developing breast cancer and is thought to be due the convergence of the accumulation of mutations together with the accumulation of senescent cells. Our working hypothesis is that senescent epithelial cells can cause preneoplastic or neoplastic changes in its neighbors, and that these changes will be manifest when cells are cultured in three dimensions, which more closely mimics the natural tissue environment than conventional two dimensional cultures. To test this hypothesis, we have successfully established two and three dimensional culture models of normal human mammary epithelial cells (HMECs) with and without a functional 16-tumor suppressor pathway. We have also created preneoplastic HMECs by introducing defined genes with oncogenic potential, particularly genes that selectively inactivate the p53 or pRB tumor suppressor pathways. We have used these, and frankly neoplastic human mammary epithelial cells, in the two and three dimensional co-culture assays in which presenescent HMECs are mixed with senescent HMECs and stromal breast fibroblasts. We have devised ways to analyze factors secreted by senescent cells and compared human mammary epithelial cells with their stromal counterparts.

15. SUBJECT TERMS
cell culture; senescent cells; mammary epithelial cells

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INTRODUCTION

Aging is the single largest risk factor for developing breast cancer and is thought to be due the convergence of the accumulation of mutations together with the accumulation of senescent cells. Our working hypothesis is that senescent epithelial cells can influence its neighbors, and that these changes will confer preneoplastic or neoplastic phenotypes on the neighboring cells. Moreover, we hypothesize that these changes will be most clearly manifest when cells are cultured in three dimensions, which more closely mimics the natural tissue environment than conventional two dimensional cell culture systems. To test this hypothesis, we proposed to establish two and three dimensional culture models of normal human mammary epithelial cells (HMECs) with and without a functional 16-tumor suppressor pathway. We also proposed to create and incorporate into these culture models preneoplastic HMECs that lack functional p53 and/or pRB tumor suppressor pathways, and frankly neoplastic human mammary epithelial cells. We proposed to monitor the cell culture models for indices of cell proliferation and differentiation, particularly morphological differentiation (ability to form structures present in normal or neoplastic breast tissue).

BODY

1. Establishment of two dimensional cell culture models of presenescent and senescent normal human mammary epithelial cells (HMECs) that retain or lack a functional p16 tumor suppressor pathway (part of approved statement of work #1).

a) In year 1, we obtained and optimized culture conditions for HMECs. We optimized culture conditions for both preselected (p16+, p16 tumor suppressor pathway intact) and postselected (p16-, p16 pathway inactivated due to spontaneous methylation of the p16 promoter) HMECs. We cultured both types of HMECs until they have undergone replicative senescence.

Research and findings: We cultured HMECs (strain 184) in mammary epithelial growth medium (MEGM), passaging continually for several weeks until there was no increase in cell number over a 2 week interval and the percentage of cells that incorporated bromo-deoxyuridine (BrdU), indicative of DNA synthesis, was <10% over a three day interval (labeling index) (replicative senescence). In two independent cultures, preselected HMECs underwent 8-12 population doublings (PDs) before cell growth ceased and the labeling index declined to 3-5%. This relatively short replicative life span of preselected HMECs makes it difficult to perform experiments with them, owing primarily to the rapid loss of presenescent cells from the culture.

In an attempt to extend the replicative life span of preselected HMECs, we also cultured them in a reduced oxygen atmosphere (3% oxygen, which more closely approaches physiological oxygen levels). Reduced oxygen made little or no difference in the number of PDs achieved before the cells arrested growth, indicating that the limited replicative life span of these cells is not due to the stress of culture in atmospheric oxygen levels (however, as discussed below, culture in 3% oxygen did affect the senescence-associated secretory phenotype). Postselected HMECs, by contrast, underwent 20-30 PDs before cell growth ceased and the labeling index declined to 8-9%. These cells therefore have a replicative life span that is adequate for many presenescent-senescent comparisons. In both types of cultures (pre- and post-selected), upon replicative senescence, 75-80% of the cells expressed the senescence marker SA-Bgal (senescence-associated beta-galactosidase).

We also explored the use of X-irradiation to induce rapid senescence of HMECs, which allowed us to study the phenotypic consequences of senescent HMECs much more efficiently than the long culture times required for replicative senescence. The phenotypes of replicatively senescent and irradiation-induced senescent HMECs were indistinguishable with respect to cell proliferation, cell morphology and expression of the senescence marker SA-Bgal.

Research and findings: Although 5 Gy X-rays are generally sufficient to induce senescence in >95% of human fibroblasts, we found that postselected HMECs were incompletely (<50%) arrested by this radiation dose. We therefore used increasingly higher X-ray doses until the cells arrested growth with little or no cell death. At 10 Gy X-ray, most (>95%) of the cells in the culture arrested growth as determined by achieving a two or three day labeling index of <10% within three days after irradiation. In addition, over an additional 3-5 days, the cells developed the typical enlarged, flattened senescent morphology. Between 75 and 80% of these irradiated cells stained positive for SA-Bgal.

b) We obtained and optimized culture conditions for mammary fibroblasts. We cultured the cells to replicative senescence, and have used X-irradiation to induce rapid senescence. Human mammary fibroblasts were similar to other human fibroblast strains in that >99% of the culture arrested growth after Gy irradiation with X-rays. As we found with HMECs, X-irradiated human mammary fibroblasts were phenotypically identical to replicatively senescent fibroblasts by the criteria of little or no cell proliferation, an enlarged morphology and SA-Bgal expression.

Research and findings: We used strain 184 human mammary fibroblasts, obtained from the same donor as the pre- and post-selected HMECs. The mammary fibroblasts grew well under culture conditions optimized for other human fibroblast strains (Dulbecco's modified Eagle's medium, 10% fetal calf serum, 4 mM glutamine). They underwent 25-30 PDs before reaching replicative senescence, as determined by cessation of growth with a three day labeling index of <10%, development of an enlarged flattened morphology, and >75% of the cells staining positive for SA-Bgal. Also similar to other fibroblast strains, human mammary fibroblasts responded to irradiation with 5 Gy X-ray by undergoing a senescence arrest within 2-3 days, showing the typical senescent morphology, low (<10%) labeling index, and high (>75%) SA-Bgal staining.

c) We established two dimensional co-cultures models in which we mixed presenescent p16+ or p16- HMECs with presenescent or senescent fibroblasts. We found that p16+ HMECs were unaffected by the presence of senescent fibroblasts in two dimensions. By contrast, p16- HMECs were stimulated to proliferate two- to three-fold by the presence of senescent fibroblasts. This finding suggests that the p16 tumor suppressor pathway (or the pRB pathway, which is positively regulated by p16), is responsible for conferring sensitivity to a senescent stromal microenvironment on HMECs. It also suggests that loss of p16 expression (by methylation or mutation) in vivo may be an important risk factor for the development of age-related breast cancers.

Research and findings: We expressed the green fluorescent protein (GFP) in pre- and post-selected HMECs using a retroviral construct we created and high titer retroviral preparations. This manipulation allowed us to quantify HMEC proliferation in mixed co-cultures with fibroblasts using quantitative fluorescence imaging. We then prepared lawns of presenescent and senescent fibroblasts on two dimensional culture dishes, replaced the medium

with serum-free medium, and seeded onto the fibroblast lawns 1 x 104 GFP-expressing HMECs, either preselected (p16 unmethylated and therefore expressed) or postselected (p16 methylated and therefore not expressed). After five days of co-culture, we quantified the amount of HMEC proliferation by measuring GFP fluorescence. The results of are shown below. HMECs, whether pre- or post-selected, proliferated similarly on presenescent fibroblast lawns (white bars). On senescent lawns (gray bars), however, postselected HMECs were selectively stimulated to proliferate. Vertical bars are standard deviations.

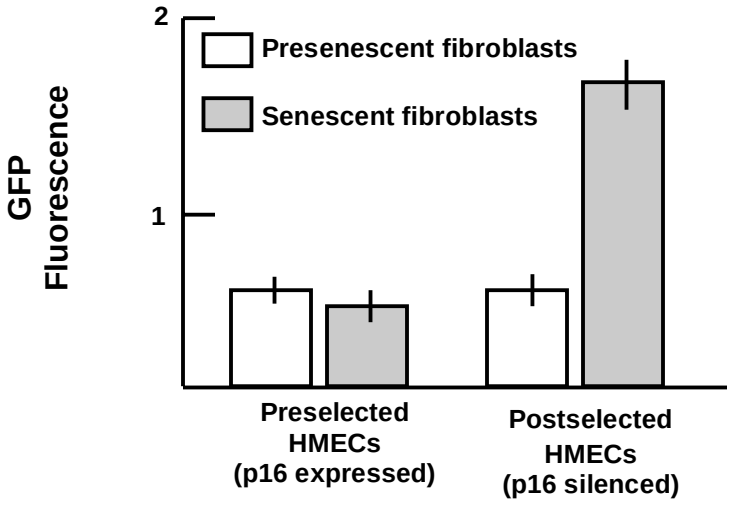


Fig 1

d) In year 1, we attempted to optimize conditions for co-culturing senescent and presenescent HMECs in two dimensions in order to obtain conditioned medium for analysis of senescence-associated secreted factors and determine the phenotypic consequences of the senescence of the epithelial cells on the their epithelial neighbors.

Research and findings: We described above the culture of HMECs to replicative senescence and the rapid induction of senescence using X-irradiation. In order to perform co-culture experiments with these epithelial cells, we must incubate them in medium containing no or minimal growth factors so that any growth stimulation we see can be attributed to the cells, not to exogenously supplied growth factors. Our initial experiments used postselected HMECs because their longer replicative life span made them easier to manipulate and because our experiments with HMECs co-cultured with fibroblasts showed that postselected HMECs were selectively sensitive to the senescent fibroblast environment.

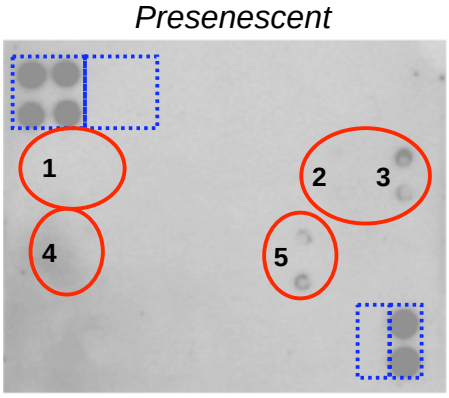
We found that neither presenescent nor senescent postselected HMECs thrived in growth factor-free medium, as determined by the gradual appearance of a vaculolated cytoplasm and gradual loss of cell adherence to the culture dish. We found that supplementing the medium with low levels (10 ng/ml) of insulin helped maintain viability, although the cells did not thrive as well as in complete medium with growth factors. In subsequent years, we continued attempts to optimize conditions for culture of HMECs in serum/growth factor-free medium.

2. In year 1, we initiated marker characterization of senescent cells using high-throughput assays of antibodies against secreted factors expressed by senescent fibroblasts and HMECs (part of approved statement of work #1).

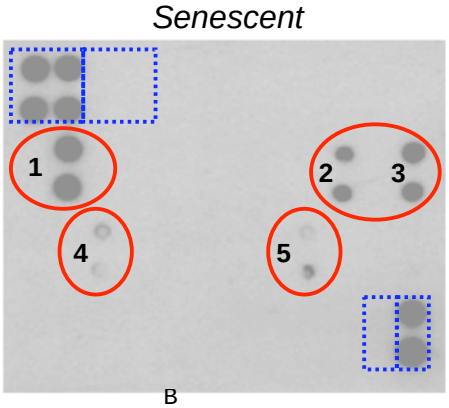
a) As proposed, high-throughput assays of expressed genes can give a much broader picture of the senescent phenotype. One such assay is the use of high-density cDNA microarrays; another, which more specifically probes the secretory phenotypes of cells, is the use of high-density antibody arrays. We chose to begin characterization using antibody arrays because our preliminary experiments indicated that these arrays have lower false positive rates, and are more pertinent to the phenotypic change we hypothesize is crucial for the effects of senescent cells on the tissue microenvironment and neighboring cells. We optimized conditions to use these arrays with chemiluminescent detection, and in a small pilot experiment have validated their use by RT-PCR and western blot analysis of conditioned medium from senescent cells.

Research and findings: The human arrays with which we initiated this study are commercially available (Raybiotech) and contain 120 antibodies directed against different human cytokines. Our initial efforts to use this technology entailed several optimization experiments for which used human mammary fibroblasts induced to senesce by X-irradiation. The results of these optimization experiments allowed us to determine 1) the number of cells and medium volumes needed to detect signals from conditioned media; 2) culture regimens to allow presenescent cells to enter a quiescent state prior to collection of conditioned medium (in order to make valid comparisons with conditioned medium from postmitotic senescent cells); 3) the need to concentrate the conditioned media 3-4 fold, and the best method to do so without detectable loss of soluble factors; 4) the intervals needed for incubation of conditioned media with the arrays, and incubations needed for chemiluminescent detection. An example of an enlarged portion of an optimized array and the preliminary result it provided is shown below. Signals outlined in blue are positive and negative controls; signals circled in red are soluble factors detected by the array, identified on the right.

Fig 2



A



B

Signal #	Identity
1	IL-6
2	MIP-1
3	MCP-1
4	KC/GRO
5	TIMP-1

The results indicate little or no change in TIMP-1 secretion (independently confirmed by RT-PCR) between presenescent and senescent cells, but significantly increased secretion of IL-6 (confirmed by enzyme-linked immunoabsorption assay), MIP-1, MCP-1 and KC/GRO by senescent cells. Because the signal intensities vary so much, chemiluminescent detection is not strictly quantitative. Nonetheless, the preliminary and semi-quantitative conclusion from these

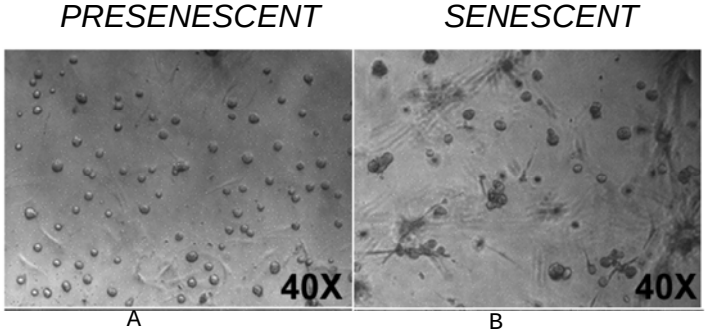
chemiluminescent-detection based arrays is that senescent cells secrete elevated levels of several cytokines that have inflammatory and/or cell proliferation enhancing and/or cell migratory activities.

3. In year 1, we also initiated establishment of three dimensional culture and co-culture model systems to evaluate effects of senescent cells on HMECs under more physiological culture conditions (part of approved statement of work #2).

a) To begin to explore the role of senescent cells on HMECs under more physiological cell culture conditions, we have begun to establish three dimensional culture models. First, we optimized conditions for alveolar morphological differentiation of HMECs, using a commercially available preparation of basement membrane components. This model is the most standard model used for studying HMEC differentiation. We showed that both p16+ and p16- HMECs formed well organized alveolar structures in this model. We also optimized conditions for ductal differentiation of HMECs, in which the HMECs are cultured in three dimensions in collagen into which fibroblasts have been incorporated. Our data suggested that the presence of senescent fibroblasts disrupted both alveolar and ductal HMEC morphogenesis. These findings suggest that a senescent stromal microenviroment may alter the morphological and functional differentiation of HMECs in human breast tissue during aging.

Research and findings: We used strain 184 HMECs, MEGM and Matrigel applied in a drip assay previously optimized for mouse mammary epithelial cells and found that pre- and post-selected HMECs formed well-ordered alveolar-like structures within 7-10 days (morphological differentiation). We used the line MCF10A, which behaves identically to strain 184 cells in the Matrigel drip assay, to explore the influence of a senescent stromal microenvironment on HMEC morphological differentiation. An example of the well-ordered alveolar structures formed in the presence of presenescent stromal fibroblasts, and the relatively disorganized structures formed in the presence of senescent stromal fibroblasts is show below.

Fig 3



4. Having established and characterized for proliferation two dimensional cell culture models of presenescent and senescent normal human mammary epithelial cells (HMECs) that retain or lack a functional p16 tumor suppressor pathway and optimized culture conditions to obtain and manipulate cultures that were either presenescent and senescent (part of approved statement of work #1), we subsequently (in year 2) explored ways to separate p16-dependent senescence from p53- and telomere-dependent senescence (also termed agonescence). We also explored culture conditions to optimize the differentiation of these cells and their ability to

participate in, and/or influence neighboring cells, in three dimensional alveolar morphogenesis (part of approved statement of work #2 and #3).

a) We explored a mechanism of cleanly separating senescence pathways in HMECs mediated by the p53 vs the p16/pRB pathway.

Research and findings: HMECs senescence owing to either of two mechanisms: 1) p16 induction by mechanisms that remain poorly understood, which engages pRB, or 2) telomere erosion and eventual dysfunction in cells that spontaneously silence p16 due to promoter methylation, which engages p53.

We used primarily HMECs strain 184, but have also used cultures from other donors, specifically donor 48 or mixed donors.

We compared cells cultured to replicative senescence, which required continual passaging for several weeks until there was no increase in cell number over a 2-week interval. At this point, cellular DNA synthesis declines to <10% over a three day interval (labeling index) and >75% of the cells express the senescence-associated beta-galactosidase (SA-Bgal), criteria for replicative senescence established in the previous funding period. We also used X-irradiation (5 Gy) to induce rapid senescence of HMECs, as described in the previous progress report. Both p16-dependent and telomere-dependent senescence results in similar loss of proliferation, DNA synthesis and SA-B-gal expression.

To determine the effect of purely p16-dependent vs purely telomere-dependent senescence, we used a dominant negative telomere-associated protein. TIN2 is a telomere-associated protein that interacts with the TRF1 telomeric DNA binding protein and is essential for telomere dysfunction. A truncated form of TIN2, termed TIN2-15C disrupts telomere function and induces immediate telomere-dependent senescence. A diagram of TIN2 and TIN2-15C is shown below, with the N-terminal, TRF1-interaction and C-terminal domains indicated.

We used TIN2-15C to induce rapid senescence in HMECs and fibroblasts. Since replicative senescence is thought to be due to both telomere dysfunction and subsequent engagement of a p53-dependent damage response and induction of the p16/pRB pathway due to stresses of unknown nature, the TIN2 mutant allowed us to induce senescence solely and synchronously by telomere dysfunction. This manipulation allows us to more cleanly separate the senescence-inducing pathways.

We created lentiviral vectors in order to efficiently deliver control and mutant TIN2 proteins to human cells. These vectors showed >90% expression in mammary epithelial and fibroblastic cells. In at least three experiments, we further demonstrated that TIN2-15C induced a telomere-dependent senescent response, as judged by DNA damage foci at telomeres, <10% DNA synthesis over three days, and approximately 80% senescence-associated beta-galactosidase (SA-Bgal) expression. The micrographs below show cells infected with a control (left micrograph) and TIN2-15C (right micrograph) vector and stained for the senescence marker SA-Bgal, which is detectable as a blue color within the cells.

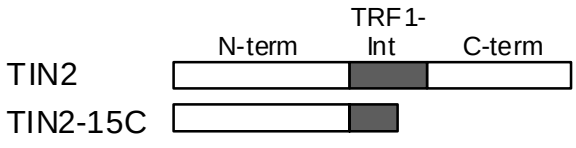
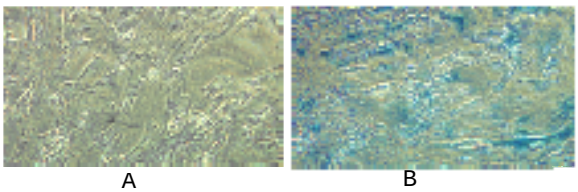


Fig 4



b) In year 2, we also defined and utilized culture conditions for HMECs, either preselected (p16+, p16 tumor suppressor pathway intact) or postselected (p16-, p16 pathway inactivated due to spontaneous methylation of the p16 promoter) HMECs, and either presenescent or senescent, using basement membrane components for induction of morphological differentiation in three dimensional cultures, as proposed. We also determined the ability of senescent HMECs to participate directly in alveolar differentiation (morphogenesis) in three dimensions.

Research and findings: We co-cultured presenescent and senescent HMECs in three dimensional alveolar morphogenesis assays, using basement membrane components and GFP-expressing cells (described above) induced to senesce by replicative exhaustion, X-irradiation or synchronous telomere dysfunction owing to expression of TIN2-15C.

In multiple attempts and over several experiments, we showed that senescent HMECs do not participate in alveolar formation per se. Greater than 95% of the alveoli formed in these three dimensional cultures were composed of presenescent HMECs, with <5% of the structures containing GFP-marked senescent cells. This is an obvious negative finding. Nonetheless, it provides an answer to one of the hypotheses set forth in our proposal – namely, do senescent HMECs disrupt normal mammary epithelial cell morphogenesis as integral parts of alveola. The answer from our experiments is no. That is, the senescence response precludes active participation in alveolar morphogenesis, and the alveolar structures are rarely if at all affected by cell-cell contacts between presenescent and senescent mammary epithelial cells.

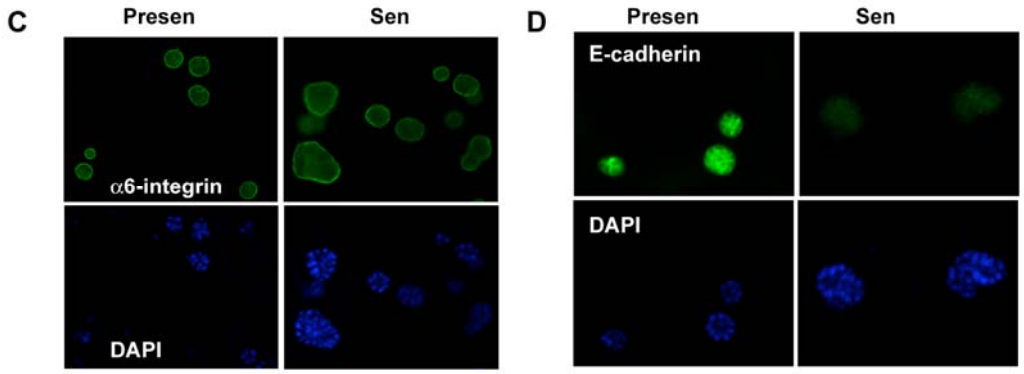
On the other hand, our negative result suggests that any effects of senescent HMECs in alveolar differentiation, then, are likely to be mediated by the diffusible factors produced by the senescent cells. This suggestion strengthens the utility of the antibody array methodologies we developed (described above and further below) to identify the specific factors secreted by cells induced to senescence by various means.

c) In year 2, we continued to characterize the effect of senescent fibroblasts in two and three-dimensional co-culture with p16+ and p16- HMECs on the differentiation characteristics of the HMECs. Our results suggest that p16- HMECs can lose proper subcellular localization of the cell adhesion protein E-cadherin in the presence of senescent but not presenescent stromal fibroblasts. These effects were most apparent when the cells were co-cultured in three, as opposed to two, dimensions. In either case, however, in the presence of senescent fibroblasts, the HMEC maintained normal expression levels and distribution of the integral membrane protein alpha-6-integrin, indicating that the epithelial cells do not lose all differentiation markers. These results suggest that a senescent stromal microenvironment may promote loss of optimal epithelial cell-cell communication in the aged breast. Since the stromal cells were not in direct contact with the epithelial cells during the co-culture, these findings further implicated secreted factors as the important mediators of the morphogenic disruption caused by a senescent stroma.

Research and findings: We immunostained co-cultures of HMECs on presenescent and senescent fibroblast lawns with an anti-E-cadherin antibody, followed by staining with a fluorescent secondary antibody. E-cadherin immunostaining is generally most prominent at sites of cell-cell junctions. Likewise, we stained with an anti-alpha-6-integrin antibody, followed by staining with fluorescent secondary antibody. In the co-cultures, E-cadherin staining was less

intense at cell-cell contact regions when postselected HMECs were cultured in the presence of senescent fibroblasts compared to culture in the absence of fibroblasts or presence of presenescent fibroblasts.

Fig 5



d) In year 2, we continued to optimize conditions to co-culture senescent and presenescent HMECs in two dimensions in order to determine the phenotypic consequences of the senescence of the epithelial cells on the their epithelial neighbors.

Research and findings: In order to perform co-culture experiments with senescent epithelial cells, we must incubate them in medium containing no or minimal growth factors so that any growth stimulation we see can be attributed to the cells, not to exogenously supplied growth factors. We found that neither presenescent nor senescent postselected HMECs thrived in growth factor-free medium, as determined by the gradual appearance of a vaculolated cytoplasm and gradual loss of cell adherence to the culture dish. We have confirmed that supplementing the medium with low levels (10 ng/ml) of insulin helps maintain viability, but this supplement was still sub-optimal. We also tried supplementing with fetuin, which we found helped maintain the viability of mouse mammary epithelial cells, but fetuin has proven insufficient to fully sustain the viability of HMECs in serum-free medium. We tried other culture manipulations, including albumin and combinations of the above additives. So far, we have not optimized conditions to our satisfaction, but we have been able to maintain HMECs for 12-16 hours in serum-free conditions, which allowed us to measure a subset of secreted factors, as described below.

5. Advanced marker characterization of senescent cells using high-throughput assays of secreted factors expressed by senescent fibroblasts and HMECs (part of approved statement of work #1).

a) As described above, we used antibody arrays to characterize the factors secreted into conditioned medium from presenescent and senescent cells. In year 2, we refined the detection and analysis methods associated with these antibody arrays, and rendered them more quantitative and reliable.

Research and findings: The human arrays with which we initiated this study (commercially available and containing 120 antibodies directed against different human cytokines) were designed for chemiluminescent detection, which has a relatively small dynamic detection range. We completed optimizing the arrays using phosphorimager detection, which is

much more quantitative and is quantitative over a much larger dynamic range. We validated selected cytokines (e.g., interleukins-6 and 8, which are highly expressed by most senescent cells) by enzyme linked immunoabsorbent assays (ELISA). In addition, we developed a method to display and analyze the array results in a format similar to that used for the display and analysis of cDNA microarrays. While we continue to add conditions and cell types to the array analyses, the results obtained thus far indicate that senescent mammary stromal cells secrete high levels of inflammatory cytokines such as interleukins-6 and 8, as well as certain matrix metalloproteinases (MMPs), particularly MMP3. In the subsequent year, we were able to apply a subset of the findings from these arrays to presenescent and senescent HMECs, an application that also required maintaining HMECs in a healthy state in serum- and growth factor-free media (since additives to the media would confound detection of factors secreted by the cells), an endeavor for which we were only partially successful (discussed later).

Shown below is a display of a part of one composite antibody array analysis in which we determined the relative secretion level of multiple factors by several different human fibroblast strains, including human mammary fibroblasts.

The signals from each antibody were quantified using a phosphorimager. The signals from the entire array were then averaged, and the quantification of signals above and below the average displayed colorimetrically. The cytokines, growth factors and other biologically active molecules that were overexpressed relative to the average are displayed as yellow. The factors that were underexpressed relative to the average are displayed as blue. Factors that did not change relative to the average appear as black.

The factors interrogated by this array are listed in the pale and dark gray boxes along the top of the display. The cells from which conditioned media were analyzed are listed in boxes to the left of the display. The cells included fibroblasts from fetal lung (WI-38, IMR90), neonatal foreskin (HCA2, BJ) and adult human mammary tissue (hBF). The red arrows indicate the results from the human breast fibroblasts. In all cases, the cells were either made senescent by X-irradiation (SEN) or were presenescent (PRE) (but quiescent owing to incubation in serum-free medium). In addition, the cultures were maintained under either standard culture conditions of ambient (approximately 20%) oxygen, or under lower physiological (approximately 3%) oxygen concentrations.

The visual display allows easy processing of the many individual data points, and led to several interesting conclusions.

First, it is clear that many factors are secreted at higher levels by senescent cells relative to presenescent cells (more yellow in the senescent samples, more blue and black in the presenescent samples). There are, however, exceptions, including factors that are secreted at a lower level by senescent compared to presenescent cells.

Second, there are somewhat more overexpressed secreted factors when cells are cultured in 20% compared to 3% oxygen. Since 20% oxygen is not physiological, despite its widespread use, and since oxidative stress can induce a senescence response, we conclude that standard culture conditions (20% oxygen) predisposes cells to express a partial senescence secretory phenotype. These results impel us to conduct future experiments using 3% oxygen culture conditions.

Third, each fibroblast strain has its own secretory phenotype, whether presenescent or senescent. This is perhaps not surprising, given the varying tissues and donor ages from which the cells were derived.

As seen in the array analysis, under physiological oxygen, human mammary fibroblasts overexpressed a variety of the growth factors and cytokines interrogated by this array, including GRO1, interleukins 8, 7, 13 and 16, as well as factors such as IGFBP2 (insulin-like growth factor binding protein 2), among others.

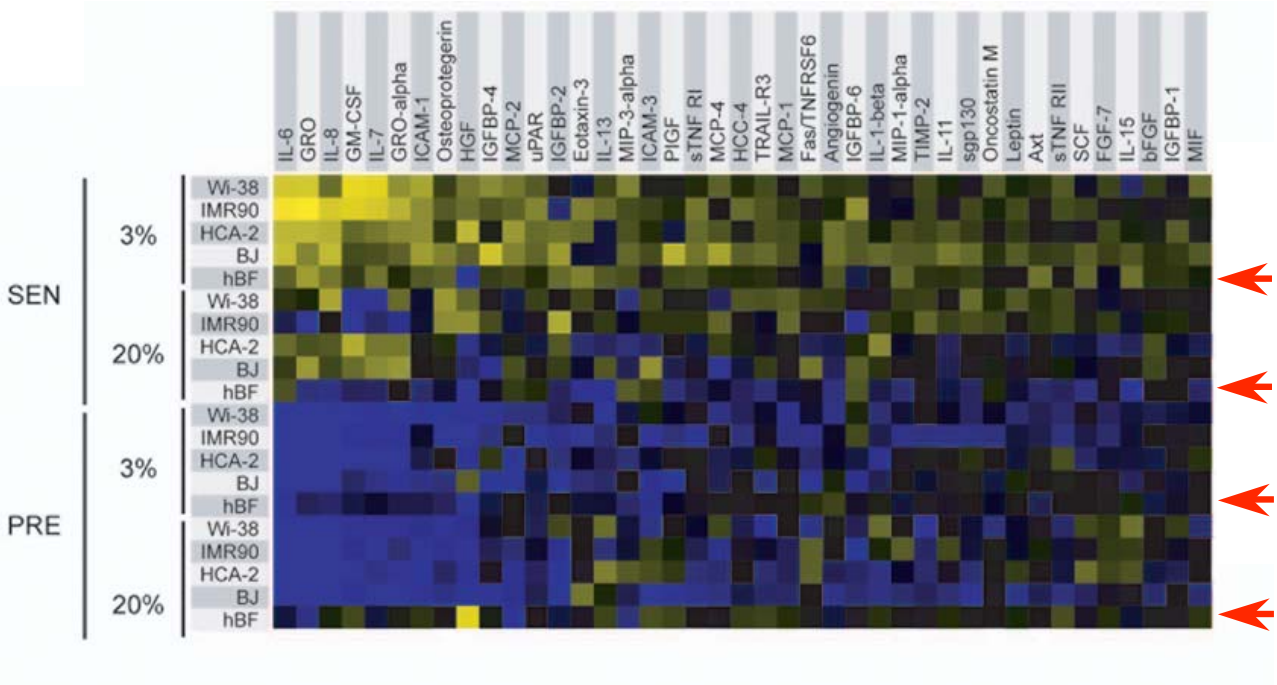


Fig 6

We validated the array analyses by both immunostaining, where it is apparent that intracellular concentrations of the cytokines increase similarly to secreted concentrations, and enzyme-linked immunoabsorbent assays (ELISAs), using selected cytokines. Results for two of the selected cytokines, interleukin 6 and 8 (IL6, IL8) are shown below for presenescent cells (PRE) and cells induced to senesce by X-irradiation (XRA) or replicative exhaustion (REP).

	pg/10^6cells	
CELLS	IL6	IL8
PRE	30	100
XRA	1000	5000
REP	2000	4000

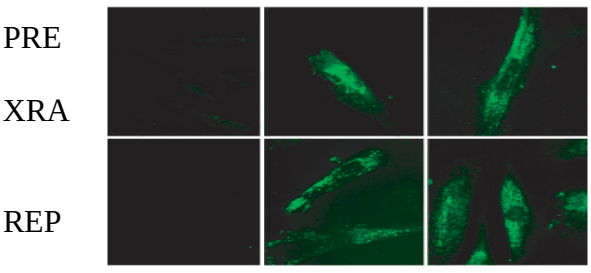


Fig 7

The ability to detect senescence-associated secreted molecules intracellularly by immunostaining suggests that at least some of these cytokines can be used as markers for the senescent state, both in culture and possibly in vivo.

6. Senescence due to TIN2-15C expression depends on p53 status in malignant breast epithelial cells (part of approved statement of work #3).

a) In year 3, an unexpected finding emerged from our experiments aimed at determining the effects of senescent HMECs on premalignant and malignant breast epithelial cells. In order to control for the senescence response per se, we attempted to induce senescence in the breast cancer cell lines we used, MCF-7, MB-MDA-231 and MB-MDA-157. As noted above in section 4a, TIN2-15C induced a senescence growth arrest in untransformed cells. Likewise, TIN2-15C induced a senescence growth arrest in MCF-7 cells. However, in the other two breast cancer cell lines, TIN2-15 appeared to induce cell death, apparently by apoptosis, rather than cell senescence. To study this phenomenon more carefully, we devised a more sensitive assay for apoptosis.

Research and findings: Most apoptosis assays (e.g., TUNEL staining, externalized phosphatidylserine (annexin V staining), nuclear fragments with a sub-G1 DNA content) capture a 'snapshot' of cells undergoing apoptosis. Since apoptosis is relatively rapid, generally taking only a few hours for completion, these assays tend to underestimate apoptosis. To circumvent this tendency, we took advantage of the fact that apoptosis by the intrinsic pathway proceeds by release of cytochrome c from mitochondria prior to activation of the execution caspases (primarily caspase 3) and proteolytic destruction of cellular constituents. We therefore immunostained cells for cytochrome c in the presence of a caspase inhibitor. Under these conditions, cells undergoing apoptosis could accumulate for 2-3 days, thereby greatly increasing the sensitivity of the determination. Cells not undergoing apoptosis showed the expected punctate staining, whereas cells undergoing apoptosis showed diffuse cytochrome c staining. The details of this assay were published in the accompanying paper: Goldstein JC, Rodier F, Garbe JC, Stampfer MR, Campisi J. 2005. Caspase-independent cytochrome c release is a sensitive measure of low-level apoptosis in cell culture models. Aging Cell 4: 217-222.

We first used this assay to determine the apoptosis rate of p16- (postselected) HMECs that had been made senescence by replicative exhaustion, a state that has been also termed agonescence. The results showed that HMECs with an intact p53 pathway undergo very little apoptosis, <1% over a three-day period. However, upon entering crisis owing to inactivation of the p53 pathway, apoptosis increased more than 10-fold over the same interval. The results from the Goldstein et al paper, in which HMECs are compared to human fibroblasts (HCA-2), are shown below.

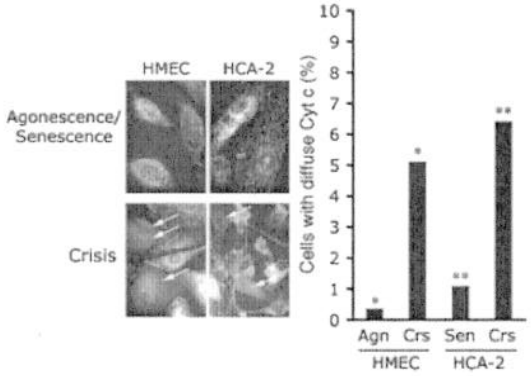


Fig 8 CICR assay detects increased apoptosis in cells in crisis. HMECs and HCA-2 fibroblasts were cultured to the end of their replicative lifespan, agonescence/senescence, and induced into crisis by p53 inactivation (HMECs) or p53 inactivation and E7 expression (HCA-2). The cells were assessed for apoptosis by CICR for a 3-day period. Arrows identify apoptotic cells (diffuse cytochrome c staining, bottom). The numbers of cells scored were 247 (agonescence, Agn), 465 (HMEC crisis, Crs), 343 (replicative senescence, Sen) and 484 (HCA-2 crisis, Crs). *$P = 0.01$ and **$P = 0.02$ in an unpaired single tailed Student's t-tests, $n = 5$ fields.

The sensitivity of breast cancer cells to telomere disruption by TIN2-15Cwere even more striking. MCF-7 cells, with wild-type p53, underwent <3% apoptosis, which increased to about 15% upon TIN2-15C expression (although MCF-7 cells are caspase deficient, this assay scores the apoptotic response prior to caspase activation). In contrast, MB-MDA-23 and MB-MDA-157 cells, which have mutant p53, underwent approximately 4-8% apoptosis in the absence of any perturbation, but apoptosis increased to >80% upon TIN2-15C expression. This unexpected finding has led us to consider the possibility – which is outside the scope of this proposal and for this reason will pursue using other sources -- that telomere disruption by TIN2 mutants or dominant-negative acting peptides might be used to selectively eliminate human breast cancer cells that have mutant or otherwise compromised p53 function.

7. The secretory phenotype of senescent HMECs is low compared to senescent human fibroblasts.

a) In year 3, we exerted considerable effort to understand and quantitatively measure the senescent secretory phenotype of HMECs, especially in relation to the senescent secretory phenotype of stromal cells.

Research and findings: While it remained difficult to maintain HMECs under the serum-free culture conditions needed for full analysis of the senescent secretory phenotype by antibody array analyses, we have been able to measure two secreted cytokines, interleukins 6 and 8(IL-6, IL-8), which are highly secreted by senescent stromal cells. For these assays, we employed quantitative ELISAs. A result is shown below. The X-axis shows the fold increase in IL-6 and IL-8 relative to quiescent fibroblasts (QFb). Senescence induced by X-irradiation showed an approximately 15- to 25-fold increase in the secretion of these cytokines in fibroblasts (XFb). Presenescent HMECs, p16+ or p16-, secreted 0.5 to 1-fold as much IL-6 and <2- to 1-fold as much IL-8, relative to the QFb control. That is, basal IL-6 ad IL-8 secretion levels were fairly similar in HMECs and fibroblasts, regardless of the HMEC p16 status. However, X-irradiation induced senescence resulted in much lower levels of cytokine secretion in HMECs compared to fibroblasts. IL-6 secretion increased about 5-fold and IL-8 secretion about 3-fold.

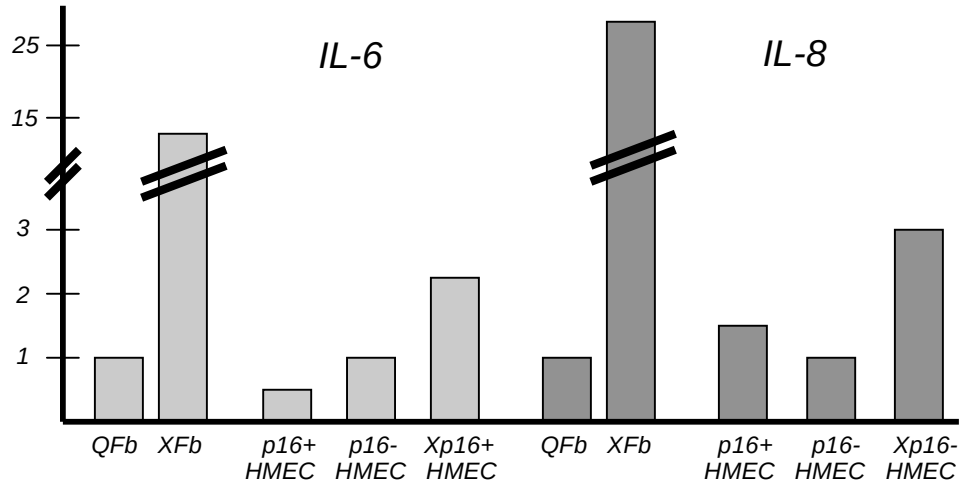


Fig 9

The levels of IL-6 and IL-8 secretion by senescent (X-irradiated) HMECs were too low to detect by immunostaining, in contrast to ready detection by immunostaining of these cytokines in X-irradiated fibroblasts, including breast fibroblasts (section 5a above).

These results suggest that although senescent HMECs arrest growth with senescence characteristics similar to fibroblasts (e.g., morphology, SA-Bgal expression), including an increase in the secretion of the inflammatory cytokines IL-6 and IL-8, the senescent secretory phenotype, at least by these criteria, is much milder in HMECs compared to stromal cells. It is therefore possible that senescence of stromal cells, compared to epithelial cells, may have a greater influence on the behavior of neighboring cells during breast cancer development.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that the relatively rapid senescence of preselected HMECs is not due to atmospheric oxygen
- Determined that HMECs permanently arrest growth with a senescent phenotype following irradiation with 10 Gy X-rays
- Determined that human mammary fibroblasts arrest growth with a senescent phenotype after 5 Gy X-rays
- Developed conditions for two dimensional co-culture models of HMECs and fibroblasts
- Determined that loss of the p16 pathway (in postselected HMECs) confers sensitivity of HMECs to senescent stromal (fibroblast) environment in two dimensional co-culture assays
- Established conditions to successfully characterize secretory phenotype of senescent cells using antibody arrays.
- Preliminary results from antibody arrays indicate increased secretion of inflammatory and cell migratory cytokines by senescent cells.
- Determined conditions for three dimensional culture models of HMEC morphogenesis.

- Determined that HMECs arrest with a senescent phenotype when synchronous telomere dysfunction is induced
- Determined that HMECs do not actively participate in alveolar morphogenesis in three dimensional culture
- Determined that senescent mammary fibroblasts can disrupt some but not all markers of differentiation in HMECs
- Optimized methods for quantification of antibody arrays to measure increased secretion of inflammatory and cell migratory cytokines by senescent cells.

- Determined p53-dependency of human breast cancer cells for apoptosis, rather than senescence, response to telomere disruption by TIN2-15C.
- Developed a highly sensitive assay for apoptosis and used the assay to measure apoptosis in irradiated HMECs and TIN2-15C expressing breast cancer cell lines.
- Determined that HMECs secrete quantitatively less of the inflammatory cytokines IL-6 and IL-8 at senescence (X-ray induced) relative to fibroblasts.

REPORTABLE OUTCOMES

Goldstein JC, Rodier F, Garbe JC, Stampfer MR, Campisi J. 2005. Caspase-independent cytochrome c release is a sensitive measure of low level apoptosis in cell culture models. Aging Cell 4: 217-222.

CONCLUSIONS

We made excellent progress in establishing the proposed two dimensional and three dimensional culture systems of p16+ and p16- HMECs with or without stromal fibroblasts, obtaining senescent cells for incorporation into two and three dimensional co-culture systems, and characterization of proliferative characteristics. We also made good progress in characterization of senescence markers in order to obtain a more comprehensive understanding of how senescent cells may alter HMEC phenotype and differentiation.

We made progress in establishing the proposed two dimensional and three dimensional culture systems of p16+ and p16- HMECs with or without stromal fibroblasts, showed that senescent HMECs do not directly participate in alveolar morphogenesis, and characterized senescence markers in order to obtain a more comprehensive understanding of how senescent cells may alter HMEC phenotype and differentiation.

We found unexpectedly that the p53-dependent telomere-dysfunction pathway of senescence is intact in normal and p53-positive cells, but telomere dysfunction, at least that caused by the mutant telomere-associated protein TIN2-15C, leads to apoptosis in cells that lack functional p53. We developed a new sensitive apoptosis assay to detect apoptosis in HMECs and other cells and used this assay to show that HMEC senescence entails very little apoptosis, and confirm the sensitivity of breast cancer cells with compromised p53 function to telomere dysfunction. We also made progress in detecting cytokines IL-6 and IL-8 secreted by senescent HMECs and conclude that the senescent secretory phenotype of HMECs is considerably milder than that of stromal cells. Whereas these cytokines can be used as senescence markers in stromal cells, they are not robust enough to be used as markers for senescent HMECs. Finally, we conclude that senescence in the stroma may constitute a greater risk to breast cancer development than the senescence of the epithelial cells.

REFERENCES

None

APPENDICES

None